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14. ABSTRACT

A significant amount of progress has been made with respect to the work described in the above proposal. We have evaluated cationic amphipathic peptide based fusion peptides (Specific Aim 1) and immunoconjugates (Specific Aim 2) for the targeted ablation of prostate cancer cells. These results have resulted in a manuscript published recently in the journal Cancer Research (see Appendix 1). Secondly, we have investigated the role of the Prostate-Specific Membrane Antigen (PSMA) in the differential intracellular sorting of fluorescent quantum dots by prostate cancer cells (Specific Aim 3). A manuscript relating to these results in currently in preparation.

Future work will involve the use of protein engineering techniques in order to generate higher efficacy peptides targeting prostate cancer cells followed by their self assembly on quantum dots in order to general multifunctional assemblies for the targeted ablation and imaging of prostate cancer disease.

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1. INTRODUCTION

ADVANCED PROSTATE CANCER DISEASE:
Prostate cancer (PCa) is the most frequently diagnosed malignancy in men in the United States with over 186, 000 cases diagnosed in 2008; approximately 30 000 men die every year due to the disease.[1, 2] Lowering androgen levels results in tumor shrinkage or decelerated tumor growth in approximately 90% of treated cases.[3] Unfortunately, these results are usually transient and a large number of patients subsequently undergo disease progression to aggressive, androgen-independent, and chemo- and radiation-therapy-resistant PCa disease. Consequently, in addition to the discovery of novel molecular therapeutics, nanoparticle-mediated ablation of PCa cells is also being currently investigated.[4, 5]

Cancer disease progression to the aggressive metastatic state is a result of the accumulation of various genetic changes. Malignant cells undergo significant differences in their cellular phenotype as a consequence of these genetic changes; characteristics include alterations in intra- and extracellular protein expression, cell polarity, and cell survival. While the polarized phenotype of non-malignant epithelial cells results in different trafficking mechanisms at their apical and basolateral regions,[6] malignant cells are typically characterized by loss in polarity, which influences intracellular sorting and trafficking of cargo in these cells.[7] In addition, due to the heterogeneous nature of epithelial tumors, phenotypic differences in cancer cells play a significant role in the uptake, intracellular sorting, trafficking, and localization of internalized cargo.[8]

PROSTATE-SPECIFIC MEMBRANE ANTIGEN (PSMA): PSMA is a ~100 kDa (750-amino acid) type II membrane glycoprotein with an intracellular segment (amino acids 1–18), a transmembrane domain (amino acids 19–43), and an extensive extracellular domain (amino acids 44–750) [9, 10]. While PSMA is also expressed by cells in the small intestine, proximal renal tubules, and salivary glands, the level of expression in these tissues is 100-1000 fold less than in prostate tissue [11-15]. Alternate splicing of PSMA results in at least three known variants of which PSM' is the most significant. PSMA is an attractive target for site-specific prostate cancer therapy for a variety of reasons. PSMA is over-expressed in human prostate cancer cells and neovasculature rendering a high degree of potential selectivity of a delivered therapeutic to tumor sites [14, 15]. PSMA is abundantly expressed in all stages of prostate cancer disease; the expression of the protein increases in cases of hormone-refractory disease and metastasis, and advanced disease. PSMA is over-expressed in tumors relative to normal cells; PSMA:PSM' ratio was found to be 9-11 in LNCaP cells, 3–6 in prostatic carcinoma, and 0.075–0.45 in normal prostate [16, 17]. Significantly for delivered therapeutics, PSMA and PSMA-antibody complexes have been show to undergo a faster rate of internalization compared to that of PSMA alone [17]. Taken together, these factors make PSMA an attractive target for delivering therapeutics specifically to advanced prostate cancer cells [18, 19].

PEPTIDE AND ANTIBODY BASED THERAPEUTICS: Peptides have been investigated for a number of disease applications [20] including anti-retroviral therapy [21], nucleic acid delivery [22], vaccines [23], antimicrobial therapy [24], and neurodegenerative diseases [25]. Peptides are attractive for anti-cancer therapy [26-29] including prostate cancer [30, 31]. Peptides can be easily synthesized biologically using recombinant means and synthetically using solid-phase techniques, with relatively low production costs. However, in most cases, peptide-based therapeutics lack the high specificites possessed by antibodies. The simultaneous display of multiple peptide copies on a molecular scaffold results in enhanced affinities of the displayed peptide due to ***polyvalency*** therefore, mitigating this limitation. Another limitation is the small molecular size of peptides which results in their rapid clearance by the reticulo-endothelial system and degradation by proteases in the body. Significant effort has therefore been devoted to increasing the half-life of peptides in vivo and modifications such as incorporation of non-natural amino acids have been employed to limit proteolysis. Antibody-based constructs have emerged as important therapeutics in several human malignancies including breast cancer, lymphoma, colorectal cancer, and lung cancer [27, 32]. Although unmodified antibodies show some therapeutic benefit, antibodies have been mainly used to specifically target cytotoxic agents including chemotherapeutic drugs, radionuclides, enzymes, and cytokines specifically to cancer cells with high efficacies.

Prostate cancer is appropriate target for antibody-based therapies for a number of reasons: (1) the prostate is a non-essential organ which allows organ- or tissue-specific antigen based targeting, (2). The common regions of prostate cancer metastasis, bone and lymph nodes, receive high levels of circulating antibodies, and (3) the small size of prostate cancer metastasis implies easier antigen access and higher penetration of antibodies. Consequently, antibody-based constructs are being investigated in prostate cancer therapy.

NANOMEDICINE:
Nanoscale therapeutics, diagnostics, and imaging agents hold great promise in the detection and treatment of advanced cancer disease.[33, 34] An understanding of the processing and fate of targeted and untargeted nanoparticles in cancer cells can facilitate the design and engineering of novel nanoscale agents that possess higher efficacies and selectivities for targeting specific intracellular locations. Receptor expression profiles on cancer cells influence the intracellular trafficking of targeted nanoparticles.[35, 36] While some reports describe the uptake of unconjugated nanoparticles,[35] most reports describe the role of conjugated molecules including polymers,[37] cell-penetrating peptides,[38, 39] and serum proteins[40] in receptor-independent (untargeted) uptake and trafficking of nanoparticles in cancer cells. Recent reports have described the role of nanoparticle size and surface chemistry on their uptake and intracellular fate in cancer cells. For example, 25-50-nm Herceptin-conjugated gold nanoparticles demonstrated the highest uptake in SK-BR-3 breast-cancer cells among nanoparticles ranging from 2 to 100 nm in size.[41] Similarly, 50-nm particles demonstrated greatest uptake in HeLa cells among unconjugated gold particles ranging from 14 to 100 nm in diameter; adsorption of serum-containing proteins on the surface of the anionic nanoparticles was thought to promote the non-specific uptake of the nanoparticles by cancer cells.[40] Polymeric particles less than 25 nm in diameter were reported to be taken up by a non-degradative, cholesterol-independent, and non-clathrin and non-caveolae-dependent endocytosis leading to their transport as punctate structures in the perinuclear region of HeLa cells; larger sized (40 nm) nanoparticles did not demonstrate this behavior.[42]

Cellular uptake of exogenous material relies on a number of different internalization mechanisms including phagocytosis, macropinocytosis, receptor-mediated endocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, and clathrin and caveolin-independent endocytosis.[43, 44] Internalized material is then sorted and trafficked to different locations inside cells depending on cellular polarity, expression of sorting and motor proteins, protein-protein interactions, and cytoskeletal organization. Genetic and phenotypic alterations can lead to pronounced differences in transport of cargo inside cells and can be both a cause and a result of a number of disease states,[45-47] including cancer.[48]

In this report, we investigate the role of cancer-cell phenotype on the uptake and intracellular routing of unconjugated anionic nanoparticles in bone-metastasis-derived PC3, PC3-flu,[49] and PC3-PSMA human PCa cells. Differences in these closely related cell lines can be indicative of phenotypic differences that occur during disease progression and different cancer-cell populations existing in tumors. These cells were employed to investigate the role of cellular heterogeneity on nanoparticle fate in cancer cells. Quantum dots (QDs) are of interest in biomedical imaging applications due to their greater photostability, broader excitation, and narrower, symmetric emission wavelengths, compared to traditional organic dyes;[50, 51] we chose QDs as model nanoparticles for our current investigation. We demonstrate that unconjugated anionic QDs are taken up spontaneously by PCa cells and that their intracellular fate is dramatically influenced by the cancer-cell phenotype.

2. HYPOTHESIS/RATIONALE/PURPOSE
The transformation of androgen-dependent disease to highly tumorigenic, metastatic, and androgen independent phenotype is a result of the accumulation of significant genetic changes. For example, overexpression of anti-apoptotic proteins (e.g. Bcl-2) leads to aggressive survival in advanced prostate cancer cells. Treatments that overcome/bypass these phenotypic changes and enhance apoptosis of cancer cells are attractive therapeutic strategies. The anti-microbial peptide KLA59 (single letter sequence: KLAKLAKKLAKLAK) has been shown to induce apoptosis in cancer cells due to its ability to depolarize mitochondrial membranes [52]. The large negative potential (-180mV) across the mitochondrial membranes [53] is the driving force that results in localization of cationic amphipathic peptides in the mitochondria. In time,

the localization of the peptide results in the depolarization of the membrane, membrane permeability transition, and release of mitochondrial contents into the cytoplasm. The release of pro-apoptotic proteins from the mitochondria including, cytochrome-c, SMAC, and AIF ultimately results in apoptosis following depolarization. Using MDPs as cancer therapeutics is attractive because the strategy acts directly on the mitochondria and avoids the effects of anti-apoptotic proteins upstream of the organelle. The inefficient delivery of therapeutics to tumor sites remains the primary obstacle in prostate cancer therapy. In particular, the preservation and damage-prevention to underlying and collateral organs such as the rectum, bladder and the urethra is important and the damage caused to nearby tissues due to inefficient targeting is cause for concern. Thus, while the use of MDPs (e.g. KLA) is attractive, strategies that direct these peptides to the diseased tissue and individual prostate cancer cells need to be developed. The hypotheses of the proposed research are summarized below:

A: PSMA is an appropriate target in advanced prostate cancer therapy. PSMA is over-expressed in prostate cancer cells and neovasculature of malignant neoplasms rendering a high degree of selectivity of a delivered therapeutic to tumor sites.

B: Antibody and peptide mediated delivery of MDPs targeted specifically to prostate cancer cells will result in the selective induction of apoptosis in these cells. The identification of cyclic peptides with micromolar (μM) binding affinities to PSMA63 (PSMA-targeting peptides; PTPs) is an opportunity to develop peptide-based targeting strategies in prostate cancer therapy. In addition, the nanomolar (nM) affinities of anti-PSMA monoclonal antibodies make it an attractive vehicle for delivering MDPs specifically to prostate cancer cells.

C: Oligomerization of PSMA-targeting peptides using molecular self-assembly can result in enhanced targeting of the PSMA. The oligomeric display of peptide epitopes using self-assembling coiled coil peptides has been demonstrated to result in mutants with higher binding affinities [54]. Using a similar approach, PTP epitopes will be displayed on oligomeric (dimeric, trimeric, and tetrameric) α-helical coiled-coil peptides as a PTP-coiled coil fusion peptide which possesses high binding affinities to the PSMA receptor.

D: The self-assembly of multiple PTP oligomers and MDPs with quantum dots will lead to multi-Dunctional constructs. It is hypothesized that the simultaneous loading of PTP oligomers and MDPs on quantum dots (QDs; 1-10nm diameter) can lead to a 'multi-functional' QD-polypeptide constructs with targeting (PTP), apoptotic (MDP) and imaging (QD) capabilities on a single platform.

Various novel therapeutic interventions, including those based on nanoparticles, are being pursued with an eye towards increasing survival in cases of aggressive, drug-resistant, metastatic, and androgen-independent PCa. We hypothesized that an investigation into the role of the advanced cancer-cell phenotype on intracellular trafficking and localization of nanoparticles can eventually aid the design of efficacious nanoscale therapeutics. As a result, we investigated the uptake, sorting, trafficking, and localization of unconjugated QDs in advanced PCa cells. While a number of cell lines, including LNCaP, C4-2, and DU-145, have been employed for the in vitro evaluation of nanoscale therapeutics for PCa,[55, 56] we chose bone-metastasis-derived PC3 cells for the current investigation. PC3 cells are androgen independent and therefore represent the advanced form of PCa disease. This is in contrast to LNCaP cells, which are androgen responsive and represent a more "manageable" form of the disease. In addition, the availability of sub-clones of PC3 cells (PC3-flu and PC3-PSMA) cells makes it convenient for investigating nanoparticle trafficking in closely related advanced PCa cells. The apoptotic efficacies of QD-polypeptide assemblies and MLN591-MDP conjugates wias evaluated using different prostate cancer cell lines. Promising leads from these experiments will be investigated in vivo using orthotopically implanted prostate cancer tumors in mice. This research will lead to valuable pre-clinical information and increase the array of site-specific therapeutics for advanced prostate cancer.

SPECIFIC AIMS

1. Generation and Characterization of Apoptosis-Inducing Quantum Dot-Polypeptide Assemblies.
2. Generation and Characterization of Apoptosis-Inducing Antibody-MDP Conjugates.
3. Parallel, Cell-Based In-Vitro Evaluation of QD-Polypeptide and MLN591-KLA Conjugates.
4. In-Vivo Evaluation of QD-Polypeptide and MLN591-KLA Conjugates.

3. KEY RESEARCH ACCOMPLISHMENTS

A significant amount of progress has been made with respect to the work described in the above proposal. We have evaluated cationic amphipathic peptide based fusion peptides (Specific Aim 1) and immunoconjugates (Specific Aim 2) for the targeted ablation of prostate cancer cells. Secondly, we have investigated the role of the Prostate-Specific Membrane Antigen (PSMA) in the differential intracellular sorting of fluorescent quantum dots by prostate cancer cells (Specific Aim 3). A manuscript relating to these results has been published in Cancer Research.[57]

We further investigated the differences in cancer-cell phenotypes can lead to significant differences in intracellular sorting, trafficking, and localization of nanoparticles. Unconjugated anionic QDs demonstrate dramatically different intracellular profiles in three closely related human-prostate-cancer cells used in the investigation: PC3, PC3-flu, and PC3-PSMA. QDs demonstrate punctated intracellular localization throughout the cytoplasm in PC3 cells. In contrast, the nanoparticles localize mainly at a single juxtanuclear location ("dot-of-dots") inside the perinuclear recycling compartment in PC3-PSMA cells, where they co-localize with transferrin and the prostate-specific membrane antigen. The results indicate that nanoparticle sorting and transport is influenced by changes in cancer-cell phenotype and can have significant implications in the design and engineering of nanoscale drug delivery and imaging systems for advanced tumors. A manuscript relating to these results is in currently in preparation.

4. REPORTABLE OUTCOMES

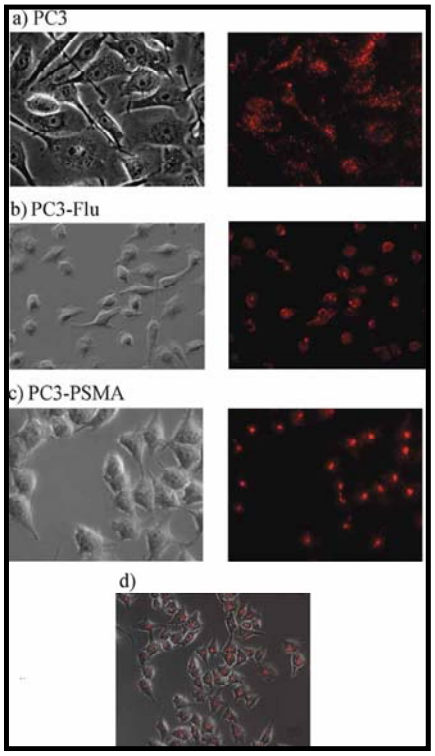

Figure 1. Differential intracellular localization of QDs in human PCa cells. a) PC3, b) PC3-flu, c) PC3-PSMA, d) overlay of phase contrast and fluorescence microscopy image of dot-of-dots formation in PC3-PSMA cells.

QDs (0.2 nM) demonstrated punctated intracellular localization throughout the cytoplasm in PC3 cells **(Figure 1a)** characteristic of lysozomal localization.[58] In contrast, QDs localized mainly at a single juxtanuclear location ("dot of dots") inside PC3-PSMA cells **(Figure 1c and d)**. Kinetic experiments indicated that the dot-of-dots formation was complete in 5 h in a concentration-dependent fashion in PC3-PSMA cells and the structure remained intact for at least 72 h (not shown). Higher concentrations of the QDs (1 nM) were required for the formation of the dot-of-dots structure in the presence of serum under similar conditions indicating that the presence of serum proteins inhibited nanoparticle uptake at lower concentrations. PC3-flu cells demonstrated trafficking profiles similar to both PC3 and PC3-PSMA cells **(Figure 1b)**; while QDs formed the dot-of-dots structure as seen in PC3-PSMA cells, they also localized throughout the cytoplasm similar to PC3 cells and along the cellular periphery.

Following the above observations, we investigated the factors that influence the uptake of nanoparticles leading to the formation of the dot-of-dots structure in PC3-PSMA cells. Different mechanisms, including lipid-raft-mediated, clathrin-mediated, and adsorptive endocytosis, play a role in the cellular entry of exogenous material. Lipid rafts are cholesterol-rich membrane platforms that have been implicated in the entry of viruses in mammalian cells. We found that extraction of cholesterol using methyl-β-cyclodextrin from the surface of PC3-PSMA cells resulted in no change in the uptake and trafficking of QDs **(Figure 2a and b)**, which indicated that disruption of lipid rafts did not inhibit the endocytosis of QDs. Clathrin-mediated endocytosis constitutes an important mechanism in the uptake of exogeneous material, including nanoparticles, in both polarized[59] and non-polarized[40] epithelial cells. Treatment with the clathrin inhibitor chlorpromazine resulted in complete inhibition of nanoparticle uptake **(Figure 2c and d)**, indicating that clathrin-mediated endocytosis was responsible for the entry of QDs in these cells.

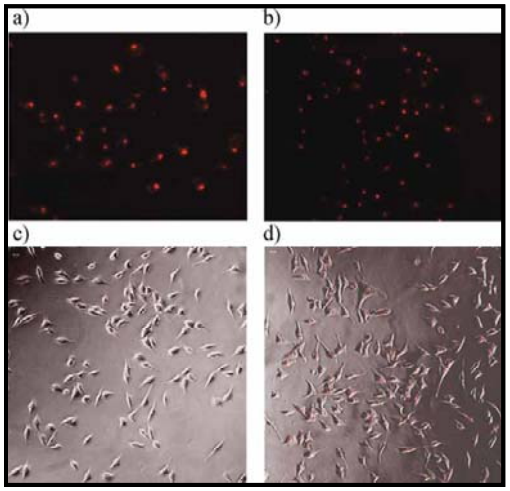

Figure 2. Role of lipid rafts and clathrin on QD internalization and dot-of-dots formation in PC3-PSMA cells. PC3-PSMA cells were treated a) with or b) without the cholesterol extracting agent, methyl--cyclodextrin for evaluating the role of lipid rafts, and c) with or d) without the clathrin inhibiting agent, chlorpromazine for evaluating the role of clathrin on the uptake of QDs.

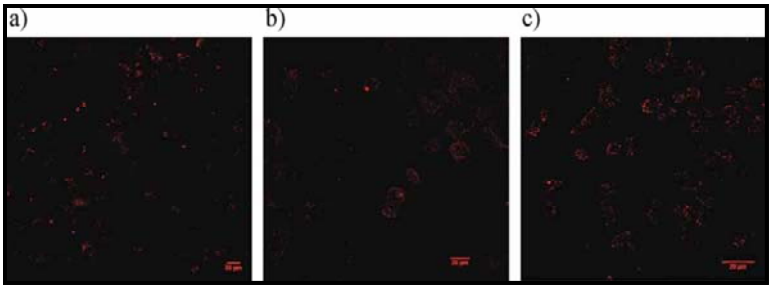

Figure 3. Effect of microtubule disruption on QD trafficking in a) PC3, b) PC3-flu and PC3-PSMA cells. Cells were treated with the microtubule polymerizing agent, nocodazole, for 1 h prior to treatment with QDs for 5 h. Scale bar = 20 μm.

Motor proteins kinesins and dyenins transport cargo-containing vesicles to the plus (cell periphery) and minus (microtubule organizing center (MTOC)) ends of microtubules, respectively. In order to investigate the role of microtubules on the formation of the dot-of-dots structure following clathrin-mediated endocytosis, we disrupted microtubule transport by treating cells with the microtubule depolymerizing agent, nocodazole. Microtubule disruption in PC3 cells **(Figure 3a)** resulted in reduced intracellular uptake and trafficking of QDs in these cells; however, the nanoparticles still localized as punctated dots throughout the cytoplasm. Nocodazole treatment resulted in complete disruption of the dot-of-dots formation in both, PC3-flu **(Figure 3b)** and PC3-PSMA **(Figure 3c)** cells, indicating that a functional microtubule network is necessary for the intracellular trafficking of nanoparticles in these cells. Interestingly, QDs were transported closer to the cell periphery and away from the nucleus in PC3-PSMA cells, indicating that microtubule disruption results in mis-sorting and altered trafficking, a phenomenon previously observed in both malignant and untransformed primary cells.[60] The punctated nanoparticle distribution in the cytoplasm of PC3-PSMA cells **(Figure 3c)** after nocodazole treatment was qualitatively similar to the nanoparticle distribution observed in PC3 cells without microtubule disruption.

Following uptake by clathrin-mediated endocytosis, molecular and nanoscale cargo are sorted in sorting endosomal complexes and are trafficked on microtubules along one of three different pathways: degradative lysozomal pathway, retrograde transport, or to the perinuclear recycling compartment (PNRC). We investigated the intracellular fate of QDs in all three PCa cell lines using confocal fluorescence microscopy. **Figure 4** shows intracellular co-localization of QDs with 4′,6-diamidino-2-phenylindole (DAPI), FITC-transferrin, Lysotracker Green DND-26, and FITC-labeled antibody (Ab) against the prostate-specific membrane antigen (PSMA), which are markers for cell nuclei, recycling endosomes, acidic compartments (late endosomes/lysozomes), and PSMA, respectively. In the case of PC3 **(Figure 4a, i)** and PC3-flu **(Figure 4a, ii)** cells, punctated dots were observed throughout the cytoplasmic space around the nucleus (shown in blue). **Figure 4a iii**, however, shows that the dot-of-dots formation (red) was found at a juxtanuclear location inside PC3-PSMA cells (nucleus shown in blue), which indicates that following uptake from the entire cell surface, QDs were trafficked along microtubules to a centralized juxtanuclear location, the MTOC, in these cells. This behavior was also observed in some PC3-flu cells **(Figure 4a, ii)**.

The dot-of-dots structure colocalized with FITC-transferrin in PC3-PSMA **(Figure 4b, iii)** and PC3-flu **(Figure 4b, ii)** cells as seen from the yellow color from an overlay of the red fluorescent QDs and green fluorescent FITC-transferrin. Transferrin is a known marker for the perinuclear recycling endosomal compartment (PNRC), which, in non-polarized cells, is a long-lived structure found close to the nucleus and near the MTOC.[60] Near-complete co-localization of transferrin and QDs indicated that the nanoparticles localized almost exclusively as

"dot of dots" in the PNRC in PC3-PSMA cells. As in the case of QDs, microtubule disruption in PC3-PSMA cells led to disruption of the PNRC and mis-sorting of transferrin. Following microtubule disruption, transferrin was trafficked to cytoplasmic compartments where only partial co-localization of the protein with QDs was seen. These results are in agreement with literature reports[60] and were qualitatively similar to those observed with PC3 cells that had not been treated with nocodazole, leading to the observation that microtubule disruption "reverted" trafficking in PC3-PSMA back to that observed in the parental PC3 cells. While some QDs co-localized with transferrin in cytoplasmic recycling endosomes in PC3 cells **(Figure 4b, i)**, a significant number of QDs did not co-localize with transferrin, indicating their localization in early/sorting endosomes in addition to cytoplasmic recycling endosomes. In the case of PC3-flu cells, vesicles containing both transferrin and QDs were seen in the cytoplasm in PC3-flu cells, in addition to the PNRC.

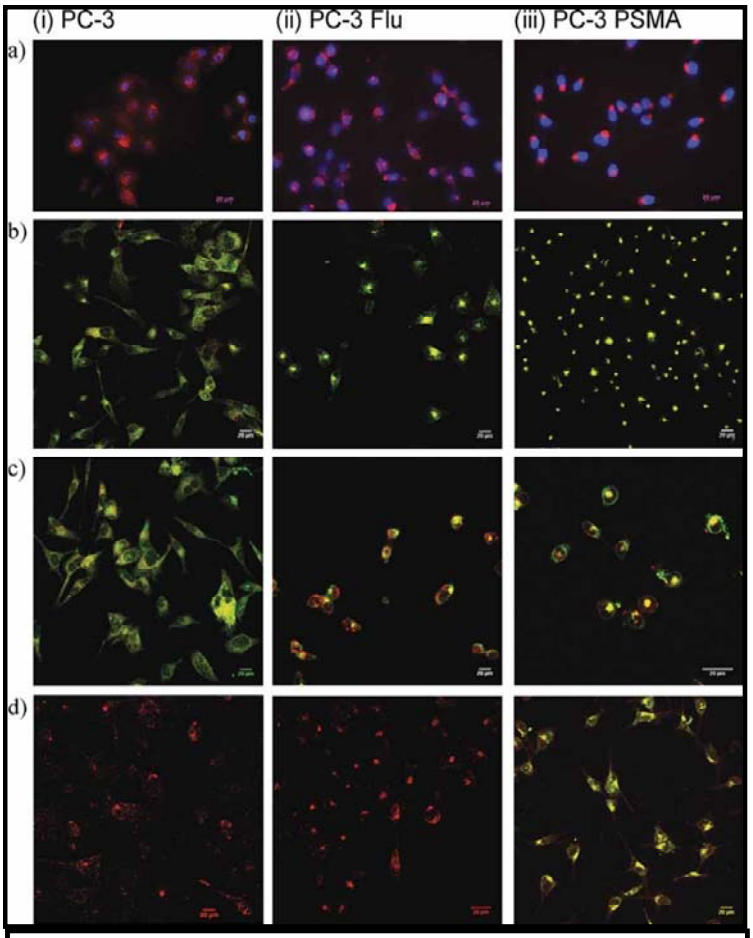

Figure 4. Intracellular localization of QDs in i) PC3, ii) PC3-flu, and iii) PC3-PSMA cells determined by co-localization for organelle/vesicle specific markers and QDs using confocal fluorescence microscopy. Confocal microscopy images show co-localization of QDs with: a) cell nuclei using DAPI (blue stain), b) recycling endosomes using FITC-transferrin, c) acidic vesicles (late endosomes and lysosomes) using LysoTracker Green DND-26, d) PSMA using FITC-labeled anti-PSMA Ab. Co-localization of green fluorescent markers (dyes) and red fluorescent QDs is shown in yellow color in the images. Scale bars = 20 µm.

Co-localization analyses were also carried out with LysoTracker Green DND-26 and all three PCa cell lines; LysoTracker stains acidic vesicles (e.g., late endosomes and lysozomes) inside cells. As with transferrin, a portion of intracellular QDs co-localized with acidic vesicles in PC3 cells **(Figure 4c, i)** further indicating the presence of these nanoparticles in different cytoplasmic compartments. Co-localization experiments also revealed the acidic nature of the QD-containing PNRC in both PC3-PSMA **(Figure 4c, ii)** and PC3-flu cells **(Figure 4c, iii)**. While some reports indicate that the PNRC is only mildly acidic with a pH range of 6.0-6.5,[3] other reports indicate that the compartment has a pH value of 5.6.[60] The latter is in agreement with LysoTracker staining of the compartment; however, we reason that the acidic nature of the cargo present in these vesicles, that is, carboxylated QDs, also contributes to the acidification of these vesicles, which in turn results in strong staining with the reagent. Interestingly, a significant fraction of QDs was also observed in non-acidic vesicles in PC3-flu cells **(Figure 4c, ii)**; the nature of these compartments is not known at this point. In contrast, while QDs were observed in the PNRC in PC3-PSMA cells, acidic compartments without QDs were observed all along the periphery of these cells.

The PSMA is a 750-amino acid type-II membrane glycoprotein, which is abundantly expressed in all stages of PCa disease; the expression of the protein increases in cases of hormone-refractory and metastatic disease. The receptor is over-expressed in approximately 70% of cases with aggressive metastatic disease and is a reliable marker of disease progression. PSMA over-expression correlates with poor prognosis[61, 62] and has been employed for the targeted ablation of PCa cells.[19, 63, 64] The extracellular region of the PSMA receptor possesses 26 and 28% homology with transferrin receptors (TfR), TfR1 and TfR2, respectively.[57, 65] Following both constitutive and Ab-binding-induced internalization from clathrin-coated

pits, PSMA is known to co-localize with transferrin in the PNRC mediated by a cytoplasmic internalization sequence and filamin.[16] Given that QDs co-localized with transferrin in PC3-PSMA cells, we asked if the nanoparticles also co-localized with PSMA in these cells. Co-localization of QDs with the FITC-labeled PSMA Ab was indeed seen in PC3-PSMA cells **(Figure 4d, iii)**, further indicating that the nanoparticles reside in the PNRC in PC3-PSMA cells. It is unclear at this point if PSMA has any role to play in the uptake and, subsequently, trafficking of QDs. It is more likely that PSMA undergoes the same fate of clathrin-mediated uptake, microtubule-mediated transport, and localization in the PNRC [66] independent of the nanoparticles. In addition, the partial localization of QDs in the PNRC of PSMA non-expressing PC3-flu cells further indicates that QD trafficking to the PNRC may occur independently of the PSMA. PC3 **(Figure 4d, i)** and PC3-flu **(Figure 4d, ii)** cells did not show staining for the anti-PSMA Ab, which is consistent with the lack of receptor expression on these cells.

5. CONCLUSIONS

In summary, our results demonstrate that unconjugated anionic QDs are spontaneously taken up by closely related cancer cells that then sort and traffic these to dramatically different fates. Serum proteins and conjugated molecules such as targeting antibodies or cell-penetrating peptides are not necessary for the uptake and trafficking of nanoparticles in these cells. Following internalization from clathrin-coated pits, nanoparticles are trafficked in vesicles along microtubules to the sorting endosomal complex in these non-polarized cells. At this stage, nanoparticles are destined for different fates depending on the cell phenotype. Nanoparticles can be either trafficked in vesicles along the "default" lysozomal degradation pathway as in PC3 cells or they can be sorted and transported along microtubules to the PNRC as observed in PC3-PSMA cells. These results underscore the importance of investigating intracellular mechanisms for delivered nanoparticles, both as therapeutics and diagnostics. Future work will elucidate molecular mechanisms underlying the decisions that lead to differential sorting of nanoparticles in different cancer cells, which, in turn, will lead to information that can be exploited to manipulate the delivery of nanoscale cargo to predetermined locations inside cells.

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Cancer-Cell-Phenotype-Dependent Differential Intracellular Trafficking of Unconjugated Quantum Dots

*Sutapa Barua and Kaushal Rege**

***A** diverse array of nanoparticles, including quantum dots (QDs), metals, polymers, liposomes, and dendrimers, are being investigated as therapeutics and imaging agents in cancer diseases. However, the role of the cancer-cell phenotype on the uptake and intracellular fate of nanoparticles in cancer cells remains poorly understood. Reported here is that differences in cancer-cell phenotypes can lead to significant differences in intracellular sorting, trafficking, and localization of nanoparticles. Unconjugated anionic QDs demonstrate dramatically different intracellular profiles in three closely related human-prostate-cancer cells used in the investigation: PC3, PC3-flu, and PC3-PSMA. QDs demonstrate punctated intracellular localization throughout the cytoplasm in PC3 cells. In contrast, the nanoparticles localize mainly at a single juxtanuclear location (''dot-of-dots'') inside the perinuclear recycling compartment in PC3-PSMA cells, where they co-localize with transferrin and the prostate-specific membrane antigen. The results indicate that nanoparticle sorting and transport is influenced by changes in cancer-cell phenotype and can have significant implications in the design and engineering of nanoscale drug delivery and imaging systems for advanced tumors.*

Keywords:
- intracellular transport
- microtubules
- nanoparticle trafficking
- perinuclear recycling compartment
- quantum dots

1. Introduction

Cellular uptake of exogenous material relies on a number of different internalization mechanisms including phagocytosis, macropinocytosis, receptor-mediated endocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, and clathrin and caveolin-independent endocytosis.[1–3] Internalized material is then sorted and trafficked to different locations inside cells depending on cellular polarity, expression of sorting and motor proteins, protein–protein interactions, and cytoskeletal organization. Genetic and phenotypic alterations can lead to pronounced differences in transport of cargo inside cells and can be both a cause and a result of a number of disease states,[4–7] including cancer.[8]

Cancer disease progression to the aggressive metastatic state is a result of the accumulation of various genetic changes. Malignant cells undergo significant differences in their cellular phenotype as a consequence of these genetic changes; characteristics include alterations in intra- and extracellular protein expression, cell polarity, and cell survival. While the polarized phenotype of non-malignant epithelial cells results in different trafficking mechanisms at their apical and basolateral regions,[9] malignant cells are typically characterized by loss in polarity, which influences intracellular sorting and trafficking of cargo in these cells.[10] In addition, due to the heterogeneous nature of epithelial tumors, phenotypic differences in cancer cells play a significant role in the uptake, intracellular sorting, trafficking, and localization of internalized cargo.[11]

Nanoscale therapeutics, diagnostics, and imaging agents hold great promise in the detection and treatment of advanced

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cancer disease.[12–14] An understanding of the processing and fate of targeted and untargeted nanoparticles in cancer cells can facilitate the design and engineering of novel nanoscale agents that possess higher efficacies and selectivities for targeting specific intracellular locations. Receptor expression profiles on cancer cells influence the intracellular trafficking of targeted nanoparticles.[15–17] While some reports describe the uptake of unconjugated nanoparticles,[18] most reports describe the role of conjugated molecules including polymers,[19] cell-penetrating peptides,[20–22] and serum proteins[23] in receptor-independent (untargeted) uptake and trafficking of nanoparticles in cancer cells. Recent reports have described the role of nanoparticle size and surface chemistry on their uptake and intracellular fate in cancer cells. For example, 25–50-nm Herceptin-conjugated gold nanoparticles demonstrated the highest uptake in SK-BR-3 breast-cancer cells among nanoparticles ranging from 2 to 100 nm in size.[24] Similarly, 50-nm particles demonstrated greatest uptake in HeLa cells among unconjugated gold particles ranging from 14 to 100 nm in diameter; adsorption of serum-containing proteins on the surface of the anionic nanoparticles was thought to promote the non-specific uptake of the nanoparticles by cancer cells.[23] Polymeric particles less than 25 nm in diameter were reported to be taken up by a non-degradative, cholesterol-independent, and non-clathrin and non-caveolae-dependent endocytosis leading to their transport as punctate structures in the perinuclear region of HeLa cells; larger sized (40 nm) nanoparticles did not demonstrate this behavior.[25]

Prostate cancer (PCa) is the most frequently diagnosed malignancy in men in the United States with over 186, 000 cases diagnosed in 2008; approximately 30 000 men die every year due to the disease.[26–28] Lowering androgen levels results in tumor shrinkage or decelerated tumor growth in approximately 90% of treated cases.[29] Unfortunately, these results are usually transient and a large number of patients subsequently undergo disease progression to aggressive, androgen-independent, and chemo- and radiation-therapy-resistant PCa disease. Consequently, in addition to the discovery of novel molecular therapeutics, nanoparticle-mediated ablation of PCa cells is also being currently investigated.[30–32]

In this report, we investigate the role of cancer-cell phenotype on the uptake and intracellular routing of unconjugated anionic nanoparticles in bone-metastasis-derived PC3, PC3-flu,[33]and PC3-PSMA[33] human PCa cells. Differences in these closely related cell lines can be indicative of phenotypic differences that occur during disease progression and different cancer-cell populations existing in tumors. These cells were employed to investigate the role of cellular heterogeneity on nanoparticle fate in cancer cells. Quantum dots (QDs) are of interest in biomedical imaging applications due to their greater photostability, broader excitation, and narrower, symmetric emission wavelengths, compared to traditional organic dyes;[34,35] we chose QDs as model nanoparticles for our current investigation. We demonstrate that unconjugated anionic QDs are taken up spontaneously by PCa cells and that their intracellular fate is dramatically influenced by the cancer-cell phenotype.

2. Results and Discussion

Various novel therapeutic interventions, including those based on nanoparticles, are being pursued with an eye towards increasing survival in cases of aggressive, drug-resistant, metastatic, and androgen-independent PCa. We hypothesized that an investigation into the role of the advanced cancer-cell phenotype on intracellular trafficking and localization of nanoparticles can eventually aid the design of efficacious nanoscale therapeutics. As a result, we investigated the uptake, sorting, trafficking, and localization of unconjugated QDs in advanced PCa cells. While a number of cell lines, including LNCaP, C4-2, and DU-145, have been employed for the in vitro evaluation of nanoscale therapeutics for PCa,[36–38] we chose bone-metastasis-derived PC3 cells for the current investigation. PC3 cells are androgen independent and therefore represent the advanced form of PCa disease. This is in contrast to LNCaP cells, which are androgen responsive and represent a more ''manageable'' form of the disease. In addition, the availability of sub-clones of PC3 cells (PC3-flu and PC3-PSMA) cells makes it convenient for investigating nanoparticle trafficking in closely related advanced PCa cells.

QDs (0.2 nM) demonstrated punctated intracellular localization throughout the cytoplasm in PC3 cells (Figure 1a) characteristic of lysozomal localization.[39] In contrast, QDs localized mainly at a single juxtanuclear location (''dot of dots'') inside PC3-PSMA cells (Figure 1c and d). Kinetic experiments indicated that the dot-of-dots formation was complete in 5 h in a concentration-dependent fashion in PC3-PSMA cells (Figure S1 and S2 in Supporting Information) and the structure remained intact for at least 72 h (not shown). Higher concentrations of the QDs (1 nM) were required for the formation of the dot-of-dots structure in the presence of serum under similar conditions (Figure S3 in Supporting Information) indicating that the presence of serum proteins inhibited nanoparticle uptake at lower concentrations. PC3-flu cells demonstrated trafficking profiles similar to both PC3 and PC3-PSMA cells (Figure 1b); while QDs formed the dot-of-dots structure as seen in PC3-PSMA cells, they also localized throughout the cytoplasm similar to PC3 cells and along the cellular periphery.

Following the above observations, we investigated the factors that influence the uptake of nanoparticles leading to the formation of the dot-of-dots structure in PC3-PSMA cells. Different mechanisms, including lipid-raft-mediated, clathrin-mediated, and adsorptive endocytosis, play a role in the cellular entry of exogenous material. Lipid rafts are cholesterol-rich membrane platforms that have been implicated in the entry of viruses in mammalian cells. We found that extraction of cholesterol using methyl-β-cyclodextrin from the surface of PC3-PSMA cells resulted in no change in the uptake and trafficking of QDs (Figure 2a and b), which indicated that disruption of lipid rafts did not inhibit the endocytosis of QDs. Clathrin-mediated endocytosis constitutes an important mechanism in the uptake of exogeneous material, including nanoparticles, in both polarized[40] and non-polarized[17,23] epithelial cells. Treatment with the clathrin inhibitor chlorpromazine resulted in complete inhibition of nanoparticle

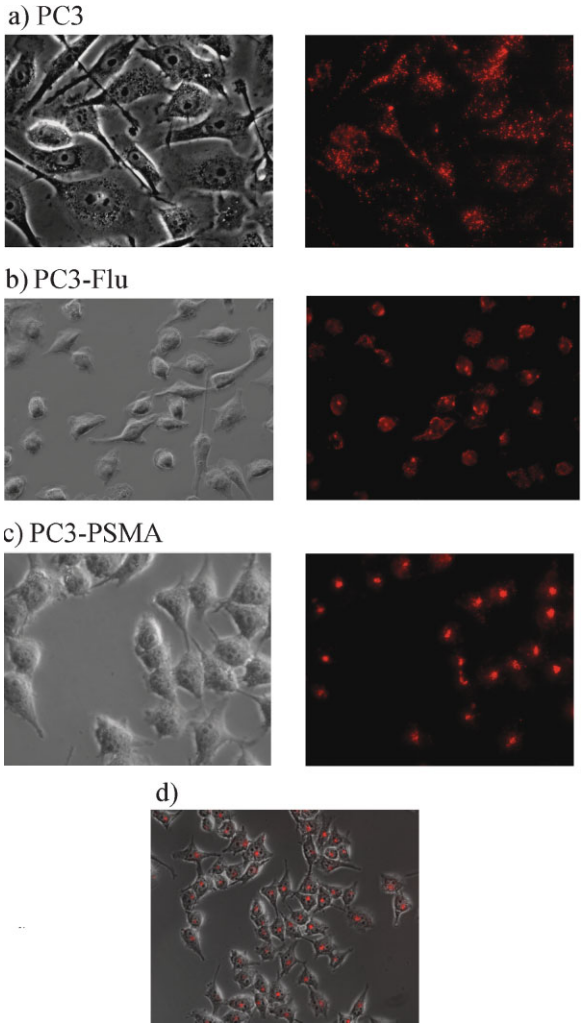

Figure 1. Differential intracellular localization of QDs in human PCa cells. a) PC3, b) PC3-flu, c) PC3-PSMA, d) overlay of phase contrast and fluorescence microscopy image of dot-of-dots formation in PC3-PSMA cells.

uptake (Figure 2c and d), indicating that clathrin-mediated endocytosis was responsible for the entry of QDs in these cells.

Motor proteins kinesins and dyenins transport cargo-containing vesicles to the plus (cell periphery) and minus (microtubule organizing center (MTOC)) ends of microtubules, respectively. In order to investigate the role of microtubules on the formation of the dot-of-dots structure following clathrin-mediated endocytosis, we disrupted microtubule transport by treating cells with the microtubule depolymerizing agent, nocodazole. Microtubule disruption in PC3 cells (Figure 3a) resulted in reduced intracellular uptake and trafficking of QDs in these cells; however, the nanoparticles still localized as punctated dots throughout the cytoplasm. Nocodazole treatment resulted in complete disruption of the dot-of-dots formation in both, PC3-flu (Figure 3b) and PC3-PSMA (Figure 3c) cells, indicating that a functional microtubule network is necessary for the intracellular trafficking of nanoparticles in these cells. Interestingly, QDs were transported closer to the cell periphery and away

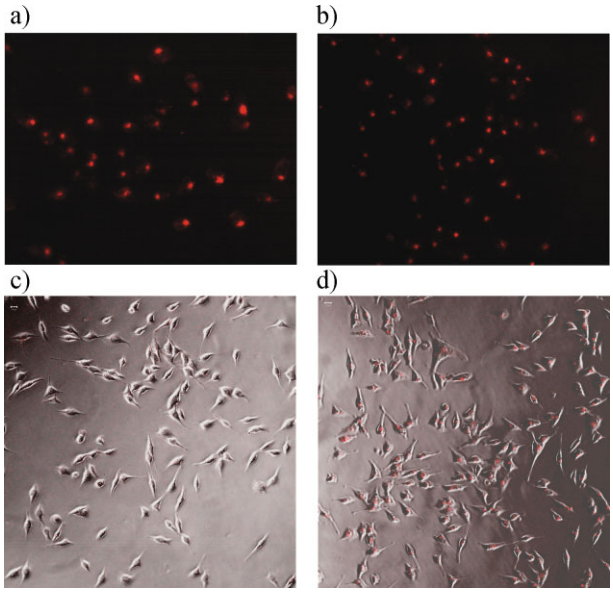

Figure 2. Role of lipid rafts and clathrin on QD internalization and dot-of-dots formation in PC3-PSMA cells. PC3-PSMA cells were treated a) with or b) without the cholesterol extracting agent, methyl-β-cyclodextrin for evaluating the role of lipid rafts, and c) with or d) without the clathrin inhibiting agent, chlorpromazine for evaluating the role of clathrin on the uptake of QDs.

from the nucleus in PC3-PSMA cells, indicating that microtubule disruption results in mis-sorting and altered trafficking, a phenomenon previously observed in both malignant and untransformed primary cells.[41,42] The punctated nanoparticle distribution in the cytoplasm of PC3-PSMA cells (Figure 3c) after nocodazole treatment was qualitatively similar to the nanoparticle distribution observed in PC3 cells without microtubule disruption.

Following uptake by clathrin-mediated endocytosis, molecular and nanoscale cargo are sorted in sorting endosomal complexes and are trafficked on microtubules along one of three different pathways: degradative lysozomal pathway, retrograde transport, or to the perinuclear recycling compartment (PNRC).[3] We investigated the intracellular fate of QDs in all three PCa cell lines using confocal fluorescence microscopy. Figure 4 shows intracellular co-localization of QDs with 4′,6-diamidino-2-phenylindole (DAPI), FITC-transferrin, Lysotracker Green DND-26, and FITC-labeled antibody (Ab) against the prostate-specific membrane antigen (PSMA), which are markers for cell nuclei, recycling endosomes, acidic compartments (late endosomes/lysozomes), and PSMA, respectively. In the case of PC3 (Figure 4a, i) and PC3-flu (Figure 4a, ii) cells, punctated dots were observed throughout the cytoplasmic space around the nucleus (shown in blue). Figure 4a iii, however, shows that the dot-of-dots formation (red) was found at a juxtanuclear location inside PC3-PSMA cells (nucleus shown in blue), which indicates that following uptake from the entire cell surface, QDs were trafficked along microtubules to a centralized juxtanuclear location, the MTOC, in these cells. This behavior was also observed in some PC3-flu cells (Figure 4a, ii).

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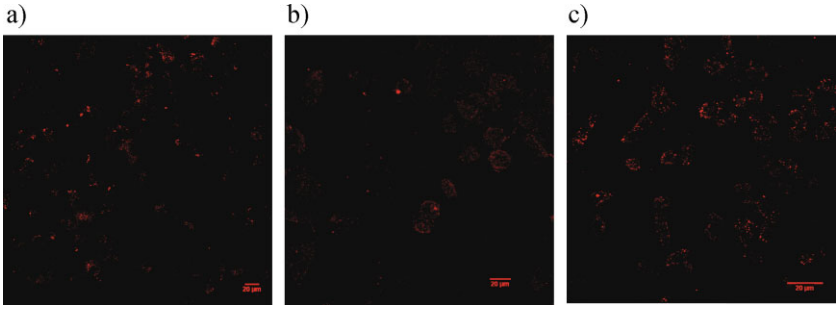

Figure 3. Effect of microtubule disruption on QD trafficking in a) PC3, b) PC3-flu and PC3-PSMA cells. Cells were treated with the microtubule polymerizing agent, nocodazole, for 1 h prior to treatment with QDs for 5 h. Scale bar = 20 μm.

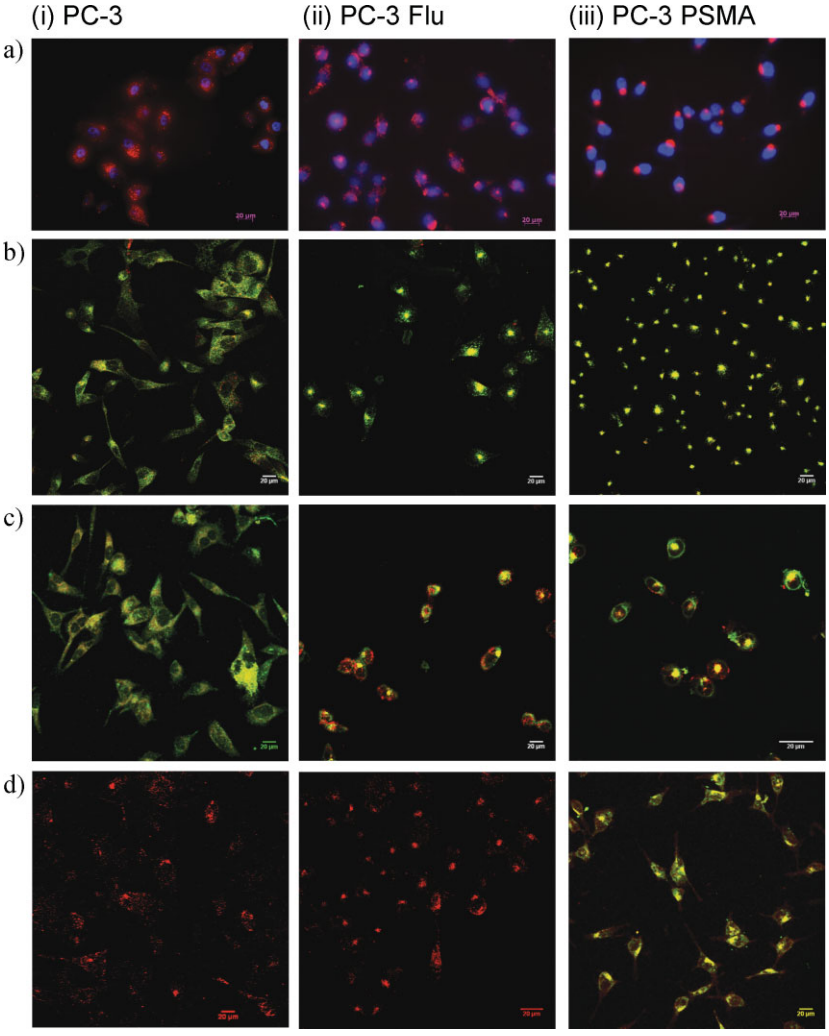

Figure 4. Intracellular localization of QDs in i) PC3, ii) PC3-flu, and iii) PC3-PSMA cells determined by co-localization for organelle/vesicle specific markers and QDs using confocal fluorescence microscopy. Confocal microscopy images show co-localization of QDs with: a) cell nuclei using DAPI (blue stain), b) recycling endosomes using FITC-transferrin, c) acidic vesicles (late endosomes and lysosomes) using LysoTracker Green DND-26, d) PSMA using FITC-labeled anti-PSMA Ab. Co-localization of green fluorescent markers (dyes) and red fluorescent QDs is shown in yellow color in the images. Scale bars = 20 μm.

The dot-of-dots structure colocalized with FITC-transferrin in PC3-PSMA (Figure 4b, iii) and PC3-flu (Figure 4b, ii) cells as seen from the yellow color from an overlay of the red fluorescent QDs and green fluorescent FITC-transferrin. Transferrin is a known marker for the perinuclear recycling endosomal compartment (PNRC), which, in non-polarized cells, is a long-lived structure found close to the nucleus and near the MTOC.[3,41,42] Near-complete co-localization of transferrin and QDs indicated that the nanoparticles localized almost exclusively as ''dot of dots'' in the PNRC in PC3-PSMA cells. As in the case of QDs, microtubule disruption in PC3-PSMA cells led to disruption of the PNRC and mis-sorting of transferrin. Following microtubule disruption, transferrin was trafficked to cytoplasmic compartments where only partial co-localization of the protein with QDs was seen (Figure S4 in Supporting Information). These results are in agreement with literature reports[41,42] and were qualitatively similar to those observed with PC3 cells that had not been treated with nocodazole, leading to the observation that microtubule disruption ''reverted'' trafficking in PC3-PSMA back to that observed in the parental PC3 cells. While some QDs co-localized with transferrin in cytoplasmic recycling endosomes in PC3 cells (Figure 4b, i), a significant number of QDs did not co-localize with transferrin, indicating their localization in early/sorting endosomes in addition to cytoplasmic recycling endosomes.[41] In the case of PC3-flu cells, vesicles containing both transferrin and QDs were seen in the cytoplasm in PC3-flu cells, in addition to the PNRC.

Co-localization analyses were also carried out with LysoTracker Green DND-26 and all three PCa cell lines; LysoTracker stains acidic vesicles (e.g., late endosomes and lysozomes) inside cells. As with transferrin, a portion of intracellular QDs co-localized with acidic vesicles in PC3 cells (Figure 4c, i) further indicating the presence of these nanoparticles in different cytoplasmic compartments. Co-localization experiments also revealed the acidic nature of the QD-containing PNRC in both PC3-PSMA (Figure 4c, ii) and PC3-flu cells (Figure 4c, iii). While some reports indicate that the PNRC is only mildly acidic with a pH range of 6.0–6.5,[3] other reports indicate that the compartment has a pH

value of 5.6.[41] The latter is in agreement with LysoTracker staining of the compartment; however, we reason that the acidic nature of the cargo present in these vesicles, that is, carboxylated QDs, also contributes to the acidification of these vesicles, which in turn results in strong staining with the reagent. Interestingly, a significant fraction of QDs was also observed in non-acidic vesicles in PC3-flu cells (Figure 4c, ii); the nature of these compartments is not known at this point. In contrast, while QDs were observed in the PNRC in PC3-PSMA cells, acidic compartments without QDs were observed all along the periphery of these cells.

The PSMA is a 750-amino acid type-II membrane glycoprotein, which is abundantly expressed in all stages of PCa disease; the expression of the protein increases in cases of hormone-refractory and metastatic disease. The receptor is over-expressed in approximately 70% of cases with aggressive metastatic disease and is a reliable marker of disease progression. PSMA over-expression correlates with poor prognosis[43,44] and has been employed for the targeted ablation of PCa cells.[37,45–48] The extracellular region of the PSMA receptor possesses 26 and 28% homology with transferrin receptors (TfR), TfR1 and TfR2, respectively.[49–51] Following both constitutive and Ab-binding-induced internalization from clathrin-coated pits, PSMA is known to co-localize with transferrin in the PNRC mediated by a cytoplasmic internalization sequence and filamin.[52] Given that QDs co-localized with transferrin in PC3-PSMA cells, we asked if the nanoparticles also co-localized with PSMA in these cells. Co-localization of QDs with the FITC-labeled PSMA Ab was indeed seen in PC3-PSMA cells (Figure 4d, iii), further indicating that the nanoparticles reside in the PNRC in PC3-PSMA cells. It is unclear at this point if PSMA has any role to play in the uptake and, subsequently, trafficking of QDs. It is more likely that PSMA undergoes the same fate of clathrin-mediated uptake, microtubule-mediated transport, and localization in the PNRC[53] independent of the nanoparticles. In addition, the partial localization of QDs in the PNRC of PSMA non-expressing PC3-flu cells further indicates that QD trafficking to the PNRC may occur independently of the PSMA. PC3 (Figure 4d, i) and PC3-flu (Figure 4d, ii) cells did not show staining for the anti-PSMA Ab, which is consistent with the lack of receptor expression on these cells.

3. Conclusions

In summary, our results demonstrate that unconjugated anionic QDs are spontaneously taken up by closely related cancer cells that then sort and traffic these to dramatically different fates (Figure 5). Serum proteins and conjugated molecules such as targeting antibodies or cell-penetrating peptides are not necessary for the uptake and trafficking of nanoparticles in these cells. Following internalization from clathrin-coated pits, nanoparticles are trafficked in vesicles along microtubules to the sorting endosomal complex in these non-polarized cells. At this stage, nanoparticles are destined for different fates depending on the cell phenotype. Nanoparticles can be either trafficked in vesicles along the "default" lysozomal degradation pathway as in PC3 cells or they can be sorted and transported along microtubules to the PNRC as observed in PC3-PSMA cells. These results underscore the importance of investigating intracellular mechanisms for delivered nanoparticles, both as therapeutics and diagnostics. Future work will elucidate molecular mechanisms underlying the decisions that lead to differential sorting of nanoparticles in different cancer cells, which, in turn, will lead to information that can be exploited to manipulate the delivery of nanoscale cargo to predetermined locations inside cells.

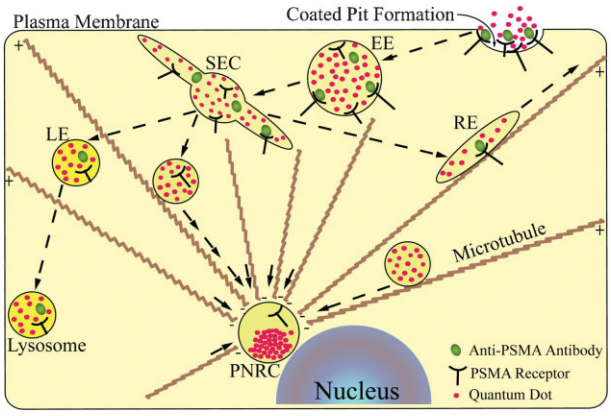

Figure 5. Schematic image of uptake, sorting, trafficking, and intracellular localization of QDs in cancer cells. Following clathrin-mediated uptake into early endosomes (EE), QDs are trafficked to the sorting endosomal complex (SEC). At this stage, vesicles are either sent back to the cell surface via recycling endosomes (RE), trafficked toward degradative late endosomes (LE) and lysozomes, or are trafficked to the PNRC near the MTOC.

4. Experimental Section

Cell culture: The PC3 human PCa cell line was obtained from the American Type Culture Collection (ATCC, VA). PC3-PSMA and PC3-flu cells, derived from PC3 cells, were generous gifts from Dr. Michael Sadelain, Memorial Sloan Kettering Cancer Center, New York. The PC3-PSMA cell line is a subclone of PC3 cells retrovirally transduced to stably express the PSMA receptor;[33] PC-3 flu cells are obtained by retrovirally transducing PC3 cells with the flu peptide.[33] All cells were grown in RPMI-1640 (HyClone, UT) containing 10% fetal bovine serum (HyClone, UT) and 1% penicillin/streptomycin (HyClone, UT) in 5% CO_2 at 37 °C in an incubator.

Quantum-dot treatment: Qdot 655 nanocrystals (ZnS-capped CdSe QDs; 8.2 μM) were purchased from Invitrogen (CA). The particle size of QDs in PBS was determined as 27 nm using dynamic light scattering; data not shown) PC3, PC3-PSMA, and PC3-flu human PCa cells were plated in 24-well cell-culture plates (Corning Inc., NY) with or without glass coverslips (12-mm circle diameter; Fisher) at a density of 50 000 cells per well and allowed to attach overnight. For uptake experiments, cells were treated with QDs (0.2 nM) in serum-free media for 5 h, fixed with 4% *p*-formaldehyde, and imaged using fluorescence microscopy (AxioObserver D1, Carl Zeiss MicroImaging Inc., Germany). The

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fluorescence excitation of the Zeiss inverted microscope was equipped with an objective of 40×/0.6 numerical aperture (NA) (without immersion) incorporating a cover glass correction ring. Images were captured using filters consisting of 550/670-nm excitation/emission for QDs, using a spot CCD camera. For kinetics experiments, cells were treated with QDs for different times, fixed, and imaged using fluorescence microscopy. Fluorescence microscopy was carried out with cells in 24-well plates using a Zeiss AxioObserver D1 (Carl Zeiss MicroImaging, Inc.) for all other experiments.

Uptake, trafficking, and intracellular localization of quantum dots: Cells (50 000/well) were treated with the lipid-raft extracting agent methyl- β-cyclodextrin (8 mM for 30 min) or clathrin disrupting agent chlorpromazine (10 μg mL^{-1} for 1 h), following which cells were washed with PBS and treated with QDs (0.2 nM) for 5 h. The microtubule depolymerizing agent, nocodazole (Sigma–Aldrich) was employed in order to investigate the role of microtubule-mediated transport of QDs in cells. Cells grown on glass coverslips placed in 24-well plates were treated with 40 μM nocodazole for 1 h at 37 °C. Nocodazole-containing medium was then removed and cells were washed with PBS and incubated with QDs for 5 h. Cells were treated with 1 μL FITC-transferrin (stock concentration 5 mg mL^{-1}; Invitrogen) for the last 1 h of the incubation in order to investigate the role of microtubule disruption on localization in recycling endosomes.

In order to visualize localization of QDs with respect to the nucleus, cells were treated with QDs for 5 h, following which cells were fixed with 4% paraformaldehyde and stained with the nucleic acid stain, DAPI (1 mg mL^{-1} stock concentration; 1.5 μL; Invitrogen), in PBS (500 μL) for 15 min at room temperature (22 °C). The cells were then washed three times with PBS and visualized using fluorescence microscopy (Zeiss AxioObserver D1 inverted microscope).

For co-localization experiments, cells were settled on coverslips placed in 24-well plates for 24 h following which QDs (0.2 nM final concentration in 500 μL) were added. After 4 h, the cells were incubated with FITC-labeled transferrin (1 μL of 5 mg mL^{-1} stock; Invitrogen), LysoTracker Green DND-26 (2 μL of 1 mM stock; Invitrogen), or FITC-labeled anti-PSMA Ab (15 μL of 50 μg mL^{-1} stock; Marine Biological Laboratory, MA) for 1 h in order to visualize recycling endosomes/PNRC, lysosomes, and the internalized PSMA, respectively. After 5 h total treatment with QDs, cells were washed twice with phosphate-buffered saline (PBS; 500 μL/well) and fixed with 4% paraformaldehyde for 10 min at room temperature (22 °C). Cells were then gently washed with PBS three times and the coverslips were removed from the 24-well plates and mounted in permount mounting media (Fisher, NJ) on glass slides for confocal fluorescence microscopy.

Laser scanning confocal microscopy was carried out with a Leica SP2 microscope (Leica Microsystems, Heidelberg, Germany) in the W. M. Keck Bioimaging Laboratory, Arizona State University, AZ in order to determine the intracellular localization of QDs. Confocal images were obtained in a z-series using an upright microscope equipped with 40×/1.25 NA oil immersion objective lens, Ar laser (488 nm) and a transmitted light detector photomultiplier (PMT). Light emitted at 525 and 650 nm was recorded for the green channel (FITC and LysoTracker) and red channel (QDs), respectively. Images were acquired with dual-channel scanning at 512 × 512 pixels using PMTs along with image acquisition and analysis software (Leica confocal software, version 2.61, Leica Microsystems, Mannheim, Germany). Images were then stacked in RGB color using Image Processing and Analysis in Java (ImageJ) 1.38X software (Rasband, W. S., ImageJ, US National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2005); the average intensity was used to compare different images.

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